Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
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ORIGINAL ARTICLE

Protective Mechanisms of Thymoquinone on Methotrexate-induced Intestinal Toxicity in Rats

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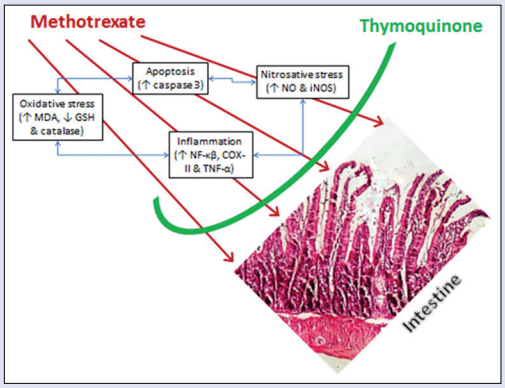
ABSTRACT

Background: Intestinal toxicity is a serious side effect in methotrexate (MTX) chemotherapy. **Objective:** To investigate the mechanisms by which the anticancer drug MTX-induced intestinal damage could be prevented by thymoquinone (TQ), an active ingredient of Nigella sativa. **Materials and Methods:** TQ was given orally for 10 days, and MTX toxicity was induced at the end of day 3 of the experiment, with or without TQ pretreatment. **Results:** MTX caused intestinal damage, represented by distortion in normal intestinal histological structure, with significant oxidative stress, exhibited as decrease in reduced glutathione concentration and catalase activity, along with significant increase in malondialdehyde level compared to control group. MTX also caused nitrosative stress evident by increased intestinal nitric oxide (NO) level, with up-regulation of inducible NO synthase expression shown in immunohistochemical staining. Furthermore, MTX caused inflammatory effects as evident by up-regulation of intestinal necrosis factor-kappa beta and cyclooxygenase-2 expressions, which were confirmed by increased intestinal tumor necrosis factor-alpha level via enzyme-linked immunosorbent assay. Moreover, MTX caused apoptotic effect, as it up-regulated intestinal caspase 3 expression. Concomitant TQ significantly reversed the MTX-induced intestinal toxic effects by reversing intestinal microscopic damage, as well as significantly improving oxidative/nitrosative stress, inflammatory and apoptotic markers tested compared to MTX alone. **Conclusion:** TQ may possess beneficial intestinal protective effects as an adjuvant co-drug against MTX intestinal toxicity during cancer chemotherapy. TQ protection is conferred via antioxidant, anti-nitrosative, anti-inflammatory, and anti-apoptotic mechanisms.

Key words: Caspase 3, inducible nitric oxide synthase, intestine, methotrexate, necrosis factor-kappa beta, thymoquinone

SUMMARY

- Methotrexate induces oxidative and nitrosative stress in intestinal tissues
- Methotrexate also initiates inflammatory and apoptotic intestinal injury
- Thymoquinone co-administration ameliorates methotrexate-induced intestinal toxicity
- Thymoquinone has antioxidative, anti-nitrosative, anti-inflammatory, and anti-apoptotic mechanisms.



Abbreviations used: COX-2: Cyclooxygenase-2, ELISA: Enzyme-linked immunosorbent assay, H and E: Hematoxylin and eosin, iNOS: Inducible nitric oxide synthase, MDA: Malondialdehyde, MTX: Methotrexate, NO: Nitric oxide, NF-κB: Nuclear factor-κB, GSH: Reduced glutathione, TQ: Thymoquinone, TNF-α: Tumor necrosis factor-alpha.

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DOI: 10.4103/0973-1296.176106

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INTRODUCTION

For more than 60 years, methotrexate (MTX) has been successfully used in treating various malignancies and autoimmune disorders.[1] Being a structural analog of folic acid, MTX inhibits folate metabolism, via interference with dihydrofolate reductase, which leads to suppression of synthesis of nucleic acid precursors; purine and pyrimidine. Unfortunately, the curative potential of MTX is sometimes accompanied by morbid multi-organ toxicity.[2] MTX may induce intestinal toxicity that might end fatally even at low MTX dosages used in the treatment of rheumatoid arthritis.[3] The mechanisms contributing to intestinal toxicity are independent of folate metabolism and involve modifying cellular metabolic processes through altering antioxidant, anti-inflammatory, and apoptotic pathways.[1] Several attempts have been made to ameliorate MTX-induced intestinal damage.[4-7] However, the outcomes were not completely satisfactory.

Thymoquinone (TQ; 2-isopropyl-5-methyl-1,4-benzoquinone) is the major bioactive component of the black seed Nigella sativa (family *Ranunculaceae*), which is considered a miracle healing herb in the middle and far East for a wide range of diseases.[8] Th s is due to the potent antioxidant, anti-inflammatory, and anti-apoptotic effects of TQ. Indeed, TQ has been shown to protect against experimental colitis.[9] In addition, TQ ameliorates inflammatory response to intestinal obstruction.[10] Interestingly, at higher doses, TQ might possess pro-oxidant cytotoxic effects that might explain its potential anticancer activity.[11] In combination with MTX, TQ was able to ameliorate MTX-induced

Cite this article as: El-Sheikh AA, Morsy MA, Hamouda AH. Protective mechanisms of thymoquinone on methotrexate-induced intestinal toxicity in rats. Phcog Mag 2016;12:S76-S81.

testicular damage.[12] The aim of the current study is to investigate the possible intestinal protective effect of TQ against MTX-induced toxicity in rats and explore the mechanisms involved.

MATERIALS AND METHODS

Chemicals

TQ was purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA), MTX from Minapharm Pharmaceuticals (Egypt), and tumor necrosis factor-alpha (TNF-α) enzyme-linked immunosorbent assay (ELISA) kit from Wkea Med Supplies Corporation (China). Kits for examining reduced glutathione (GSH) and catalase were purchased from Biodiagnostic (Giza, Egypt). The ready-to-use inducible nitric oxide (NO) synthase (iNOS), nuclear factor-κB (NF-κB/p65), cyclooxygenase-2 (COX-2), and caspase 3 rabbit polyclonal antibodies were purchased from Thermo Fisher Scientific Inc./Lab Vision (Fremont, CA, USA).

Experimental design

Thi ty-two adult male rats of 180–220 g weight were purchased from the National Research Center (Giza, Egypt). All animal care and experimental procedures were in accordance with European (EU) directive 2010/63/EU. Rats were housed as four rats per cage in the standard animal facility during the whole experiment, where they had free access to commercial laboratory chow and tap water. Animals were left to acclimatize for 2 weeks before the start of the experiment. Afterward, animals were weighed and divided into four groups (n = 8 each). TQ-treated group received a single daily oral dose of 10 mg/kg/day TQ by gastric gavage for ten consecutive days.[13] MTX-treated group received a single i.p. dose of 20 mg/kg MTX[14] at the end of day 3 of the experiment. Combined MTX/TQ-treated group received both MTX and TQ treatments as previously indicated. Untreated group served as control.

Sample preparation

After 7 days of MTX injection, total body weights of rats were recorded at the end of the experiment. Rats were sacrificed and venous blood samples were collected from the jugular vein, centrifuged at 5000 rpm for 15 min. Serum was then collected and stored at −80°C until used. The small intestine was removed and the jejunoileal segment (10 cm from the Treitz ligament till 10 cm from ileocecal junction) was divided into two sections. One jejunoileal section had its mucosa scraped off, snap frozen in liquid nitrogen, and kept at −80°C until further use. The second section was fixed in 10% formalin and embedded in paraffin for histopathological and immunohistochemical examinations. Mucosal samples were homogenized in 20% w/v ice-cold phosphate buffer (0.01 M, pH 7.4). The homogenate was centrifuged at 3000 rpm for 20 min and the supernatant was aliquoted to avoid sample thawing and refreezing, and was kept at −80°C until used.

Evaluation of intestinal tissue oxidative stress markers

Biochemical oxidative stress markers were determined in intestinal mucosal tissue homogenate, where GSH concentration, catalase activity, and lipid peroxide content were evaluated. Spectrophotometric kits were used for the assessment of GSH level and catalase activity and the results were expressed as μmol/g tissue and unit/g tissue, respectively. Tissue content of lipid peroxides was determined by biochemical assessment of thiobarbituric acid reacting substance through spectrophotometric measurement of color at 535 nm.[15] The results were expressed as equivalents of malondialdehyde (MDA) in tissue homogenate in nmol/g tissue.

Assessment of nitrosative stress marker and tumor necrosis factor-alpha in intestinal tissue homogenate

For the assessment of nitrosative stress in rat intestinal mucosal homogenate, the stable oxidation end products of NO, nitrite, and nitrate were used as an index of NO production, as NO has a half-life of only a few seconds, being readily oxidized to nitrite then to nitrate. The method used was based on Griess reaction,[16] which depends on conversion of nitrate into nitrite by copperized cadmium granules, then measuring the total nitrites spectrophotometrically at 540 nm. Results were expressed as nmol/100 mg tissue. TNF-α was determined according to ELISA kit manufacturer's instructions.

Histopathological and immunohistochemical examination

Five μm thick paraffin sections of intestinal specimens were prepared and then routinely stained with hematoxylin and eosin dyes. Stained slides were microscopically analyzed using light microscopy. For immunohistochemical staining, sections were fixed at 65°C for 1 h. Trilogy pretreatment (deparaffinization, rehydration, and antigen unmasking) was used to enhance standardization of the pretreatment step and produce more consistent results. The sections were incubated with ready-to-use rabbit polyclonal antibodies against iNOS, NF-κB, COX-2, and caspase 3. After applying the antibodies, slides were incubated overnight at 4°C followed by 20 min of PolyHRP enzyme conjugation. Afterward, diaminobenzidine chromogen was applied for 2 min, and then rinsed, followed by counterstaining with Mayer hematoxylin before examination under the light microscope. Using image J 1.41 (freeware; rsbweb.nih.gov/ij), the immunopositive cells and total number of cells in a field was calculated.[17] Results were the average of counting three sections from each rat and were expressed as percent of immunopositive cells compared to total number of cells.

Statistical analysis

The data were analyzed by one-way ANOVA followed by Dunnett Multiple Comparison Test. The values are represented as means ± standard error of mean. All statistical analyses were done using GraphPad Prism version 5.00 (San Diego, CA, USA). The differences were considered signifi ant when $P < 0.05$.

RESULTS

Effect of thymoquinone on intestinal histopathology in methotrexate-treated rats

Intestinal histopathological examination revealed that control and TQ groups had normal structure of villi and crypts [Figure 1a and b, respectively]. MTX-treated group, on the other hand, presented with degenerated villi and flattened crypts, with focal loss of intestinal epithelial cell lining [Figure 1c]. Treatment with MTX/TQ improved intestinal histology, with only mild shortening of villi [Figure 1d].

Effect of thymoquinone on oxidative stress markers in methotrexate-treated rat intestine

GSH concentration, catalase activity, and MDA level were determined as markers of oxidative stress in intestinal mucosa. MTX-treated group

showed a signifi ant decrease in GSH concentration and catalase activity compared with untreated control [Table 1]. Concomitant treatment with MTX and TQ increased intestinal GSH and catalase values to levels statistically higher than the MTX-treated group. On the other hand, intestinal MDA levels increased in the MTX-treated group compared to control. Th s increase was reversed by combined treatment with MTX/TQ which showed signifi antly lower levels of MDA compared to the group treated with MTX alone.

Effect of thymoquinone on nitrosative stress markers in methotrexate-treated rat intestine

Total nitrite/nitrate levels, as well as expression of iNOS, were assessed as indicators of nitrosative stress. Total nitrite/nitrate levels increased in the MTX-treated group compared to control, which was reversed in MTX/TQ-treated group, which showed signifi antly lower levels compared to the group treated with MTX alone [Table 1]. Similarly, TQ-treated group did not show any signifi ant difference in iNOS intestinal expression [Figure 2b] compared to control [Figure 2a], while MTX treatment caused a signifi ant increase in expression [Figure 2c]. Pretreatment with TQ prior to MTX signifi antly decreased iNOS intestinal expression compared to MTX alone [Figure 2d]. The

Table 1: Effect of TQ on intestinal oxidative/nitrosative stress markers in MTX-induced intestinal toxicity in rats

Groups	Control	TQ	MTX	MTX/TQ
GSH (μmol/g tissue)	5.5±1.2	5.7±0.4	2.6±0.2[a]	5.1±0.3[b]
Catalase (U/g tissue)	10.1±0.4	10.9±0.8	4.9±0.3[a]	9.4±0.8[b]
MDA (nmol/g tissue)	34±1	32±2	72±5[a]	51±4[b]
Nitrite/nitrate (nmol/0.1 g tissue)	41±2	38±2	106±7[a]	58±5[b]

Animal groups tested are control group, animals treated with TQ alone, animals treated with MTX alone, or with MTX together with TQ pretreatment. Values are representation of 8 observations as mean±SEM. Results are considered signifi antly different when $P<0.05$. [a]Signifi ant difference compared to control, [b]Signifi ant difference compared to MTX group. TQ: Thymoquinone; MTX: Methotrexate; GSH: Reduced glutathione; MDA: Malondialdehyde; SEM: Standard error of mean

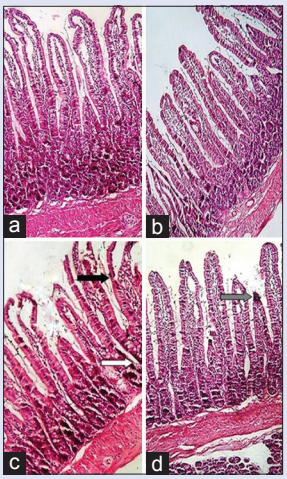

Figure 1: Intestinal histopathological picture of rats treated with methotrexate, thymoquinone, and their combination. A photomicrograph of a section in rat intestine (H and E, ×100) of: (a and b) Control and thymoquinone-treated groups, respectively, showing a normal microscopic picture of intestinal villi. (c) Methotrexate-treated group shows intestinal architectural distortion with degenerated villi (black arrow) and crypt flattening (white arrow). (d) Methotrexate/thymoquinone group shows restored normal intestinal morphology, with only mild shortening of intestinal villi (gray arrow)

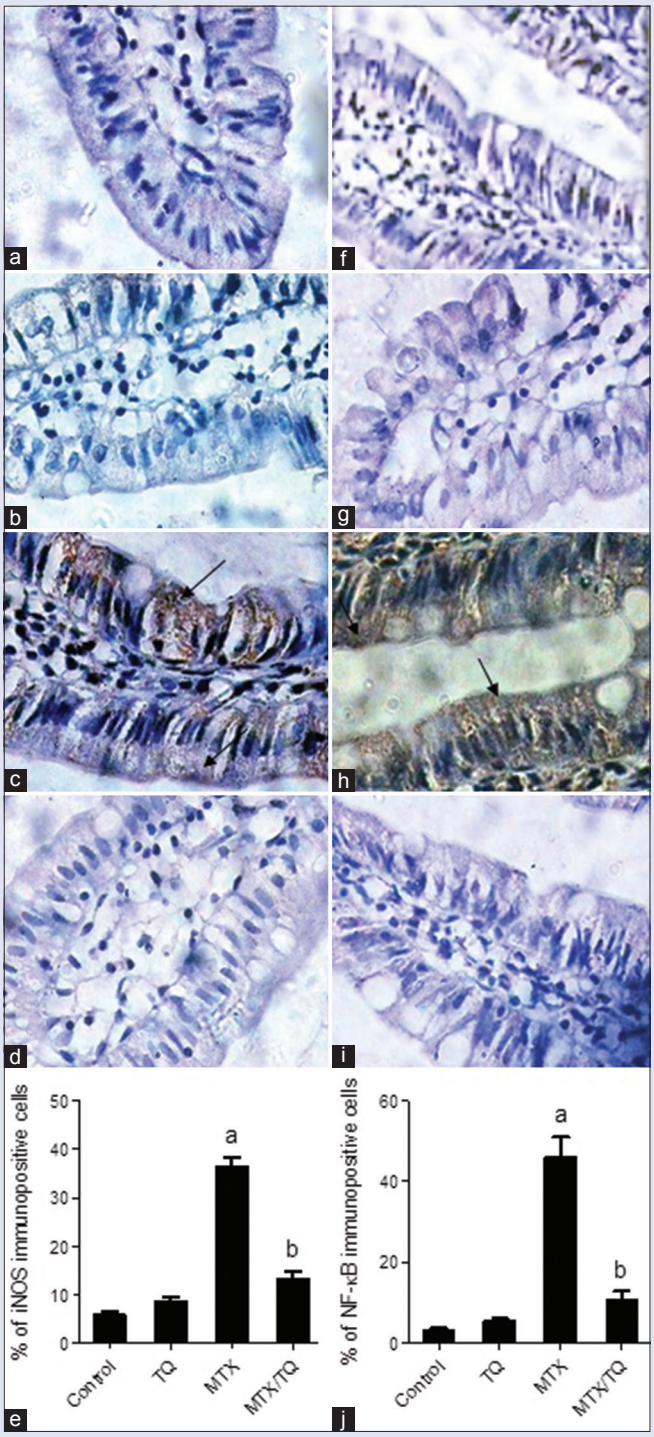

Figure 2: Effect of thymoquinone on inducible nitric oxide synthase and nuclear facto-κB expression in methotrexate-treated rat intestine. Localization of inducible nitric oxide synthase (left panel) and nuclear factor-κB (right panel) was assessed by immunohistochemical staining (×400) of: (a and f) Control, (b and g) thymoquinone-treated, (c and h) methotrexate-treated, and (d and i) combined methotrexate/thymoquinone-treated groups. Arrows point at areas of up-regulated cellular expression. (e and j) represent semi-quantitative analysis of the results for inducible nitric oxide synthase and nuclear facto-κB expression, respectively. Values represent means ± standard error of the mean of percent of immunopositive cells for the respective marker per field of each animal, 3 fields/animal. Significant difference is reported when $P < 0.05$. [a]Significant difference compared with control and [b]significant difference compared with methotrexate group

signifi ance was calculated through semi-quantitative analysis of the results of iNOS expression [Figure 2e].

Effect of thymoquinone on inflammatory markers in methotrexate-treated rat intestine

To evaluate the effect of TQ on inflammatory pathways, the level of the pro-inflammatory cytokine; TNF-α, in intestinal mucosa, as well as intestinal expression of NF-κB and COX-2 were evaluated. The level of TNF-α in MTX-treated group signifi antly increased compared to control group, while in MTX/RES group, TNF-α signifi antly decreased compared to rats treated with MTX alone [Figure 3]. Expression of NF-κB and COX-2 was visualized via immunohistochemical staining [Figure 2; right panel and Figure 4; left panel, respectively] and semi-quantitative analysis was further performed to evaluate the degree of signifi ance [Figures 2j and 4e, respectively]. Expression of both NF-κB and COX-2 in TQ-treated group [Figures 2g and 4b, respectively] was comparable to background expression in control groups [Figures 2f and 4a, respectively]. To the contrary, the MTX-treated group showed signifi antly higher expression of NF-κB and COX-2 [Figures 2h and 4c, respectively] compared to respective controls. Pretreatment with TQ prior to administration of MTX caused a signifi ant decrease in expression of both markers compared to MTX alone [Figures 2i and 4d, respectively]. The signifi ance was calculated through semi-quantitative analysis of the results of caspase 3 expression [Figure 4j].

Effect of thymoquinone on expression of caspase 3 as marker of apoptosis in methotrexate-treated rat intestine

Immunostaining of rat intestine using caspase 3 antibody [Figure 4; right panel] was performed as a marker of apoptosis. Both control and TQ groups showed minimal caspase 3 expression [Figures 4f and 4g, respectively]. In the MTX-treated group [Figure 4h], however, caspase 3 was signifi antly up-regulated, especially in the epithelial lining the villi. Th s expression was signifi antly reversed in the MTX/TQ group [Figure 4i].

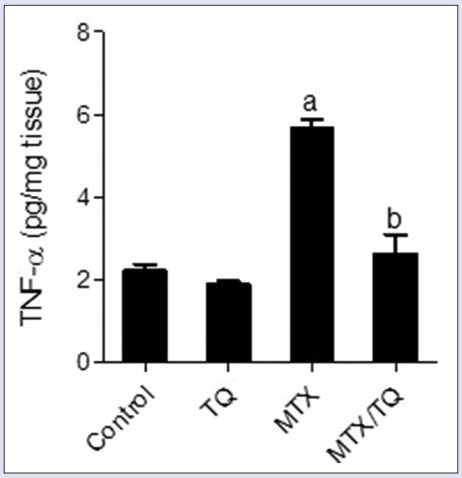

Figure 3: Effect of thymoquinone on intestinal tumor necrosis factor-alpha in methotrexate-induced intestinal toxicity in rats. Animal groups tested are control group, animals treated with thymoquinone alone, animals treated with methotrexate alone, or with methotrexate together with thymoquinone pre-treatment. Values are representation of 8 observations as means ± SEM results are considered significantly different when $P < 0.05$.[a] Significant difference compared to control, [b] significant difference compared to methotrexate group

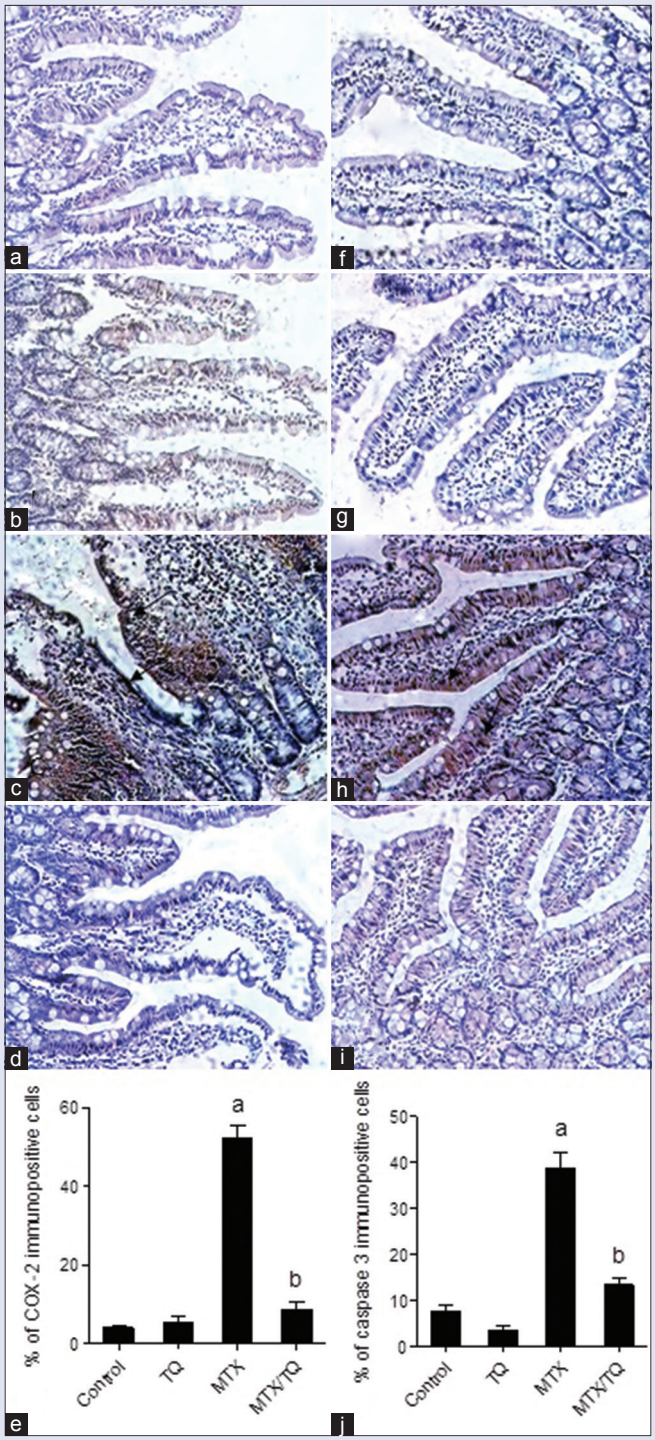

Figure 4: Effect of thymoquinone on cyclooxygenase-2 and caspase 3 expression in methotrexate-treated rat intestine. Localization of cyclooxygenase-2 (left panel) and caspase 3 (right panel) was assessed by immunohistochemical staining (×200) of: (a and f) Control, (b and g) thymoquinone-treated, (c and h) methotrexate-treated, and (d and i) combined methotrexate/thymoquinone-treated groups. Arrows point at areas of up-regulated cellular expression. (e and j) represent semi-quantitative analysis of the results for cyclooxygenase-2 and caspase 3 expression, respectively. Values represent means ± standard error of the mean of percent of immunopositive cells for the respective marker per field of each animal, 3 fields/animal. Significant difference is reported when $P < 0.05$. [a]Significant difference compared with control and [b]significant difference compared with methotrexate group

DISCUSSION

Using MTX may be accompanied by damage of intestinal mucous membranes and mucositis, which may limit the patients' ability to endure treatment, hinder their nutritional status, and might even end fatally.[3] Following MTX administration, mucositis is initiated by the generation of reactive oxygen species as a response to MTX-induced DNA and non-DNA damage, followed by up-regulation of transcription factors including NF-κB and subsequent activation of genes of pro-inflammatory cytokines producing proteins, such as TNF-α, which cause direct tissue damage and promote apoptosis.[18] In concurrence with this model of mucositis, the present study demonstrated that MTX increased intestinal mucosa oxidative/nitrosative stress markers, up-regulated NF-κB and COX-2, increased TNF-α level, and induced apoptosis as illustrated by the induction of caspase 3 expression.

Few studies reported that the main constituent of Nigella sativa; TQ, may confer protection against MTX toxicity. One study reported that TQ has a protective effect against MTX-induced testicular toxicity.[12] Another study, in a different animal model than the present study, suggested that TQ protects against MTX-induced renal damage.[19] Here, we proved that TQ provides intestinal protective activity against MTX-induced mucositis. The vast majority of studies showing alimentary tract protection of TQ were conducted on the stomach.[20-23] Only few studies focusing on such protection was carried out on the intestine, as in experimental acetic acid-induced colitis,[9] trinitrobenzene sulfonic acid-induced colitis,[24] and intestinal obstruction.[10]

In the current study, we explored the mechanisms involved in TQ-conferred intestinal protection and found that they include reversal of oxidative and nitrosative stress, down-regulation of inflammatory markers as NF-κB and COX-2, as well as inhibition of apoptosis. The antioxidant characteristics of TQ have been previously reported in different organs, as in the forebrain,[25] kidney[26,27] heart,[28] prostate,[29] and liver.[30,31] TQ was also known to modulate NO level and iNOS expression in different tissues.[26,32] The anti-inflammatory/anti-apoptotic effects of TQ have also been documented in several previous studies.[30,33]

Recently, a review[34] about interaction among oxidative, nitrosative, inflammatory, and apoptotic pathways showed how complicated it is to determine if the relationship among these pathways is a cause or a consequence of one another. Still, we hypothesize that the powerful antioxidant activity of TQ prevents MTX-induced oxidative stress from initiating intestinal damage, which, in turn, prevent the activation of TNF-α/NF-κB/COX-2 inflammatory pathway, as well as the subsequent triggering of automated cell death; apoptosis.

The protective effect of TQ against MTX-induced toxicity raises the question whether it confers similar protection to cancer cells, decreasing MTX chemotherapeutic efficacy. TQ has been reported to possess potential anticancer activity by itself on intestinal tumor development in $Msh2^{loxP/loxP}$ Villin-Cre mice[35] and on several tumor cells, including human hepatocellular carcinoma,[36] breast cancer,[37] and lung cancer.[38] In addition, TQ showed synergistic effects when given prior to conventional anticancer drugs as cisplatin[39] and doxorubicin.[40] Still, further studies are necessary to confi m lack of interference or possible synergism between TQ and MTX on tumor cells.

CONCLUSION

The main constituent of Nigella sativa; TQ, confers intestinal protection against MTX-induced mucositis. The mechanisms involved include interference with MTX-induced oxidative/nitrosative stress, inflammation, and apoptosis.

Financial support and sponsorship

Th s study was supported in part by Deanship of Scientific Research (DSR, Grant no. 150201), King Faisal University, Saudi Arabia.

Conflicts of interest

There are no confli ts of interest.

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